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Managing Devil Facial Tumour Disease in Tasmanian Devils (*Sarcophilus harrisii*): An investigation of heat shock proteins as potential vaccine adjuvants

Monika Payerhin
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Managing Devil Facial Tumour Disease in Tasmanian Devils (*Sarcophilus harrisii*):
An investigation of heat shock proteins as potential vaccine adjuvants



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Abstract

The world's largest carnivorous marsupial, the Tasmanian devil (*Sarcophilus harrisii*), is facing extinction from a deadly, highly communicable cancer that has already decimated over 85% of devil populations in the wild: devil facial tumour disease (DFTD). DFTD cells effectively evade recognition by the immune system, and every devil that contracts the disease dies from it. Many attempts have been made at developing a vaccine that could help save this now-threatened species. Heat shock proteins have been linked to enhanced immune recognition of pathogens, making them potential candidates for acting as adjuvants to such a vaccine against DFTD. In this study, the effect of heat shocking DFTD cells on HSP expression was assessed. DFTD cells were heat shocked at 40°C for varying lengths of time, with the maximum being 24 hours. RNA expression was determined for HSP 27, HSP 60, HSP 70, and HSP 90, and relative protein expression was determined for HSP 70 and HSP 90. HSP 27 was shown to have significantly increased relative RNA expression after heat shocking when compared with untreated cells. Relative RNA expression for HSP 60 was not found to be significant even after heat shocking. Expression of HSP 70 increased significantly after being heat shocked for several hours. Accordingly, the relative RNA expression for HSP 70 increased with length of time of heat shocking. Finally, HSP 90 showed insignificant variation in protein expression when compared with untreated cells, even after heat shocking; the same was found to be true of its relative RNA expression. Heat shock proteins 27 and 70 show the most potential in being used to initiate an immune response against DFTD cells in devils.

Keywords: animal pathology; immunology; veterinary science; *Sarcophilus harrisii*; devil facial tumour disease; heat shock proteins

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1.0 Introduction

1.1 Background

Tasmania has long been known for its unique flora and fauna, many of which are endemic to the island. Among these is a species known as the Tasmanian devil (*Sarcophilus harrisii*), the largest carnivorous marsupial in the world. This is a title that *S. harrisii* acquired less than a century ago with the extinction of the Tasmanian tiger (*Thylacinus cynocephalus*). Now, *S. harrisii* is facing the same fate as the *T. cynocephalus* faced, but for a very different reason: a relatively recently discovered infectious cancer that is decimating populations of devils in the wild. Known as devil facial tumour disease (DFTD), this fatal cancer has led to the species being listed as threatened in Tasmania, with the possibility of extinction looming in the not-so-distant future (Hawkins et al., 2006).

1.2 Spread and Manifestation of DFTD

Devil facial tumour disease is a highly communicable cancer that has already eliminated 85% of the population of *S. harrisii* in the wild (Norrie, 2012). The disease was first observed in northeastern Tasmania in 1996. Since then, it has spread throughout most of Tasmania, thus far avoiding only the northwestern part of the island. Even in 2007, population studies indicated that DFTD was present in at least 60% of the Tasmanian mainland, which was likely an underestimate due to the difficulty of monitoring the disease's spread in more remote areas (McCallum et al., 2007). Several locations in Tasmania have been extensively surveyed since the arrival of the disease, including the Freycinet Peninsula (Lachish et al., 2007) and Mt. William (Hawkins et al., 2006), both on the east coast. Researchers found that population decreases as drastic as 60% and 75%, respectively, of *S. harrisii* had occurred since the disease was first noted in these areas. The adult survival rate of devils in some areas including the Freycinet

Peninsula has dropped to nearly zero (McCallum et al., 2007). If this trend continues throughout the rest of Tasmania, extinction of *S. harrisii* may soon be a reality rather than just the threat it was previously believed to be.

DFTD manifests itself in the form of large malignant tumours on the face and neck of devils affected by the disease. The tumours grow uncontrollably, often blocking vision, respiratory tracts, and the mouth area. Every devil that has ever been recorded with the disease has died from it, resulting in a mortality rate of 100% for animals with DFTD. Most die from starvation because of the obstruction of their ability to feed, as the tumours invade areas in and around their mouths. Additionally, if the devils survive long enough, the tumours often metastasize to internal organs; this can lead to organ failure and secondary infections (Murchison et al., 2010).

1.3 Origin and Transmission of DFTD

Research indicates that DFTD originated from a single Schwann cell in a female *S. harrisii*, called patient zero. This cell developed into a cancer cell and divided out of control to become a tumour, a mass of cancer cells. The same cells that were present in that original tumour have been in every *S. harrisii* that has since contracted the disease (Woods, 2014). This is unusual in cancers and is due to the fact that the cancer cells themselves are the infectious agents; DFTD is transmitted by allograft. An allograft is a tissue transplant from one animal to another of the same species, though the genetic makeup of the two animals is not identical (Pearse and Swift, 2006). The tumours are primarily transmitted between *S. harrisii* individuals when they bite each other during fights and the tumour cells from an infected devil are implanted into the healthy devil's face. They also commonly contract the disease after feeding on the same roadkill that a diseased devil fed on (Hawkins et al., 2006).

The most compelling evidence for the allograft theory of transmission is the similarity in the karyotypes and genotypes of the tumours. Each of the tumours examined in a study conducted by Pearse and Swift (2006) had 13 chromosomes as opposed to the usual 14 in *S. harrisii*, and both sex chromosomes were missing. The chromosomes that were present were abnormal and mutated: one chromosome 6 and both chromosomes 2 were missing, and the long arm of one chromosome 1 was absent. Among all of the tumours studied, there were no intermediate stages between the normal arrangement of chromosomes and the arrangement of chromosomes in tumours, indicative of the fact that the same tumour cells are implanted into each individual that has the disease rather than the tumours developing independently in each individual (Pearse and Swift, 2006). A study conducted by Murchison et al. in 2010 supports this theory; the researchers found that the genotypes of each tumour studied were very similar to each other but were distinctly different from the genotypes of both their hosts and of unaffected individuals (Murchison et al., 2010).

1.4 Role of MHC

Because DFTD kills every animal infected with the disease, it is evident that DFTD cells are not recognized by the immune systems of devils. Tumour cells effectively evade the immune system and are not rejected at any point during their growth. The mechanism for this has been recently investigated, and researchers have found that DFTD cells do not express cell surface major histocompatibility complex (MHC) molecules (Siddle et al., 2013). MHC molecules are essential for proper immune function in vertebrates, as they bind to peptide fragments from pathogens and present them to the immune system for recognition by T-cells. T-cells then destroy and kill the tumour cells (Alberts, 2002). MHC molecules, when activated and working properly, can even induce an immune response by acting as antigens themselves and spurring the

production of antibodies. MHC molecules are generally responsible for transplant rejection, which does not occur in DFTD cells. In fact, DFTD cells seem to do the opposite by down-regulating genes necessary for the processing of antigens (Siddle et al., 2013). *S. harrisii* has very limited genetic diversity of MHC sequences, which in turn leads to restricted function of the immune system in recognizing antigens on tumour cell surfaces (Morris et al., 2012). A study conducted by Morris et al. in 2012 examined MHC diversity in devils that inhabited Australia before European settlement; the researchers found that low diversity in devil MHC sequences has been in *S. harrisii* populations for roughly 6,000 years. Moreover, they believe that this low genetic diversity is responsible for the numerous population crashes experienced by devils in the past and for their unusual susceptibility to diseases like DFTD (Morris et al., 2012). A study conducted by Lane et al. (2012) revealed that devils from north-western Tasmania, where DFTD has not yet infiltrated the population, had far higher diversity at their MHC genes than did devils from eastern Tasmania, where the disease has affected the majority of the population. This is further evidence that MHC sequences with low genetic diversity among individuals are linked with higher susceptibility to diseases including DFTD (Lane et al. 2012). The inability of MHC molecules in *S. harrisii* to effectively present DFTD antigens to T-cells for destruction has led to interest in finding ways to improve MHC antigen processing. Molecules known as heat shock proteins have been investigated as possible enhancers of MHC antigen recognition and presentation to T-cells.

1.5 Heat Shock Proteins

Heat shock proteins (HSPs) are evolutionarily highly conserved proteins that are present in every cell and are essential for proper cell function. There are many families of HSPs, all of which fall under the bigger protein group of molecular chaperones. Molecular chaperone

proteins work to ensure that protein chains fold into their proper conformations after translation, which is crucial for determining and performing their functions (Suzue et al., 1997). HSPs are well known for their ability to protect cells and their proteins from stressful conditions such as heat, pH, and inflammation (Van Eden et al., 2013). Equally important, however, is the role of certain HSPs in the immune system. Research done by Suzue et al. (1997) elucidates the role of heat shock fusion proteins in immune function. Heat shock fusion proteins are heat shock proteins derived from pathogens and covalently linked to ovalbumin, a protein that can stimulate an allergic reaction in study subjects. The heat shock fusion proteins examined in this study were found to function as deliverers of antigens to MHC presentation pathways. They were described as being “promising candidates for vaccines...in populations of MHC-disparate individuals,” (Suzue et al., 1997) including *S. harrisii*. Further, a more recent study conducted by Tobian et al. in 2004 found that bacterial heat shock proteins enhanced MHC processing and presentation of antigens to T-cells. The HSPs were shown to increase antigen peptide uptake, which in turn resulted in enhanced MHC presentation of the antigen to the immune system. Tobian et al. (2004) also mentioned the potential of bacterial heat shock proteins in vaccines intended to enhance the presentation of antigens to T-cells for killing. Segal et al. (2006) followed this by discussing the possibility of purifying HSPs from tumour cells along with antigenic peptides from the same tumour cells. The HSPs could then be used as a vaccine to present the isolated tumour-specific peptides to the T-cells within the immune system, forcing the immune system to recognize the antigenic peptides as foreign and presenting them for destruction. Finally, a study conducted by Li et al. (2006) showed that mice immunized with a vaccine containing heat shock protein complexes survived being injected with tumour cells in much higher numbers than did non-immunized mice. The heat shock protein complexes that were tested were shown to produce

a specific anti-tumour immunity. Even heat shock proteins alone were shown to induce non-specific immunity (Li et al., 2006). With the upregulation of HSPs in DFTD cells could come increased effectiveness in MHC presentation of tumour cells to T-cells for destruction. The purpose of this study was to investigate the possibility of using HSPs as adjuvants to a vaccine intended to initiate an immune response against DFTD cells.

1.6 Research Questions

The questions investigated in this study were: are heat shock proteins 27, 60, 70, and 90 present in DFTD cells? Can they be upregulated by heat shocking them, and which length of incubation time results in the highest level of upregulation? Can these heat shock proteins be considered as potential DFTD vaccine adjuvants?

1.7 Justification for Study

This study is extremely important and urgently required, because DFTD has already wiped out as much as 85% of the population of Tasmanian Devils in the wild. It is a highly transmissible cancer that has a 100% mortality rate – no devil infected will survive. With a rapidly decreasing number of healthy devils found in Tasmania, it is crucial to develop a method for immunizing the animals that have not yet been affected by the disease. Although preliminary vaccines have been created, none have been successful in initiating an immune response strong enough to prevent a tumour from forming. An adjuvant is needed to enhance the effect of the vaccine, and the heat shock proteins investigated in this study could hold the key. The findings of this study could be a step toward designing a more effective vaccine against DFTD and ultimately to saving the threatened *S. harrisii*.

1.8 Study Aims

This study was part of an ongoing project at Menzies Institute for Medical Research at the University of Tasmania with the overarching goal of developing a vaccine that can immunize Tasmanian Devils against DFTD and save the species from extinction. More specifically, this study aimed to investigate the possibility of using heat shock proteins as adjuvants to this vaccine. The plan for this study was first to determine whether heat shock proteins 27, 60, 70, and 90 are present in Tasmanian Devil Facial Tumor Disease (DFTD) cells. Then, if they were found to be present, the next goal was to upregulate these proteins in DFTD cells by heat shocking the cells for varying lengths of time; the purpose of this was to identify the ideal conditions for maximal protein upregulation and minimal cell death. Heat shock proteins are important for an immune response, and because DFTD seems to be able to evade Tasmanian devils' immune systems, the upregulation of HSPs could result in a stronger immune response against DFTD cells. Preliminary methods for heat shocking the HSPs of interest were based on Dokladny et al. (2006) but were modified in further experiments.

2.0 Methods

2.1 Study Site

This study took place at Menzies Institute for Medical Research, an institution of the University of Tasmania in Hobart, Tasmania. The facilities make up what is known as the University of Tasmania Medical Science Precinct. The institute was established in 1988 and has since become a world-class medical research facility, participating in both clinical and basic science research. Menzies Institute for Medical Research was the ideal location for this study for several reasons. Its geographic proximity to areas of Tasmania that have been ravaged by DFTD

made it convenient for sample collection from diseased devils and for testing the effectiveness of immunization with preliminary vaccines. Additionally, the institute is able to work closely with and receive funding from the Save the Tasmanian Devil Program, which is also located in Hobart. Finally, the superior facilities at Menzies Institute for Medical Research and extensive previous research done there on DFTD made this research possible

2.2 Study Organisms

2.2.1 *Sarcophilus harrisii*

The Tasmanian devil, *Sarcophilus harrisii*, is a carnivorous marsupial of the family Dasyuridae, found in the wild only in Tasmania, Australia. They are distributed throughout all of Tasmania, living both in forested areas and in more urban areas. Devils are characterized by their wiry black-brown fur with occasional white patches, and they are usually the size of a small dog. They tend to inhabit hollowed logs and caves and are most active at night. Tasmanian devils are fierce hunters, feeding mostly on birds, snakes, small mammals, and often on roadkill. Pound for pound, they have one of the strongest bites of all mammals because of their muscular jaws. Devils are solitary and protective of their territories, earning their names because of such behaviors as lunging, baring their teeth, snarling, and biting at encroaching devils or other predators (National Geographic, n.d.). Despite having seemingly healthy immune responses, the species has an unusual flaw in their immune systems in regards to not rejecting devil facial tumour disease cells that are implanted into their bodies (Woods, 2014). This makes them an appropriate study species for this project investigating the use of heat shock proteins to enhance an immune response against cancer cells.

2.2.2 Heat Shock Proteins

Heat shock proteins are highly conserved intracellular molecules that are responsible for several important cell functions, including the folding and transfer of proteins and the induction of immune responses to pathogens. They are particularly important for the presentation of antigens to T-cells and for the activation of these T-cells (Segal et al., 2013).

The heat shock proteins studied in this project are HSP 27, HSP 60, HSP 70, and HSP 90. HSPs are named and grouped into families based on their molecular masses in kilodaltons (kDa); HSP 27 is in the small heat shock protein family and is 27 kDa, HSP 60 is in the HSP 60 family and is 60 kDa, HSP 70 is in the HSP 70 family and is 70 kDa, and HSP 90 is in the HSP 90 family and is 90 kDa (Calderwood et al., 2006). All four of these HSPs have similar functions: they recognize proteins that are denaturing due to stressful environmental conditions, and they protect them from aggregation while aiding in their refolding. Their important function in the immune system is to assist the MHC in presenting antigens to T-cells for destruction. Heat shock proteins were chosen to be studied as potential vaccine adjuvants because they have shown promising results in previous studies in enhancing MHC antigen processing and presentation to T-cells. HSPs are a good possibility for acting as adjuvants in an anti-DFTD vaccine because there is generally no autoimmune reaction of the individual against HSPs derived from pathogens. This is due to the fact that HSPs from pathogens and the corresponding proteins in mammals can be as much as 50% identical at the amino acid level, and the immune system does not recognize the pathogen-derived HSPs as “non-self” (Lamb et al., 1989). Heat shock proteins 27, 70, and 90 in particular were chosen for this study because HSP 70 and HSP 90 are known to be present in DFTD cells and HSP 27 is thought to be present. These HSPs have been well

studied in other organisms and there is much literature describing their function in the immune system. HSP 70 specifically has been shown to be highly immunogenic, or effective at initiating an immune response, and HSP 90 has been shown to be slightly immunogenic (Udono and Srivastava, 1994). Additionally, they were convenient to use because antibodies against each of these HSPs were available. Most importantly for this study, HSP 27, HSP 70, and HSP 90 were all found to be increasingly expressed with heat treatment in a study conducted by Dokladny et al. (2006) using a method similar to the one used in this study.

2.3 Western Blot to Determine Presence of HSP 27 and HSP 70

Western blot analysis was used to detect heat shock protein 27 in DFTD cells. Protein previously extracted from DFTD cells and stored at -80°C was prepared with LDS buffer, a reducing agent, and deionized water. A total of 10 µL of this solution was loaded into each well of a gel (4%-12% Bis-Tris Protein Gel; Life Technologies) along with a pre-stained protein ladder. The gel was allowed to run for 35 minutes with MES buffer at a constant voltage of 165 V. The gel was then dry-transferred onto a nitrocellulose membrane for 7.5 minutes (iBlot Dry Blotting System; Life Technologies) and washed with a blocking buffer solution (1X iBind Solution; Life Technologies). The Western blot was then performed: the membrane was then placed on an iBind card in the iBind Western Device (Life Technologies) with the protein-side down. A total of 2 mL of the diluted primary antibody [mouse anti-human HSP 27 (1/1,000)] was added to the first well in the device, 2 mL of the blocking buffer were added to the second well, 2 mL of the diluted secondary antibody [goat anti-mouse HRP (1/1,000)] were added to the third well, and 6 mL of the blocking buffer were added to the fourth well. The membrane was allowed to incubate for 2.5 hours and was then rinsed in distilled water for 2 minutes. The membrane was then stained with a chemiluminescent substrate solution (Immobilon Western

Chemiluminescent HRP Substrate; Merck Millipore) and incubated for 5 minutes. Finally, the membrane was imaged for 2 minutes using Carestream Molecular Imaging with the setting Chemiblot: standard signal. The procedure for just the Western blot was repeated for HSP 70 the following day to detect HSP 70 using the same membrane; the primary antibody used was mouse anti-human HSP 70 (1/1000) and the secondary antibody used was goat anti-mouse HRP (1/5000).

2.4 Heat Course Trials for RNA Extraction – Version 1

DFTD cells (C5065 cell line) that had been growing in culture were collected, centrifuged at 300 x g for 10 minutes at room temperature, and re-suspended in 6 mL of complete media [RPMI medium (RPMI 1640, USA) + 10% fetal calf serum + 5% GlutaMAX + 5% Antibiotic-antimycotic (Anti-Anti); Gibco]. Six 1.5 mL tubes (Eppendorf Safe-Lock Microcentrifuge Tubes) were filled with 800 μ L of this solution each. The cell concentration was 2.8×10^6 cells per mL, or 2.24×10^6 cells per 800 μ L. Each of the tubes of cells was designated to be heated for a certain number of hours: one for zero (0) hours, one for two (2) hours, one for four (4) hours, one for eight (8) hours, one for eighteen (18) hours, and one for twenty-four (24) hours. All of the tubes except the 0 hour tube were placed in a heating block set to 41°C. The tubes were individually taken out of the heating block after they incubated for their designated amount of time, staggering the rest of the procedure; for the 0 hour tube, the following steps were started immediately without any incubation. Right after removal from the heating block, the cell viability for each tube was checked. The tubes were then centrifuged at 14,000 rpm in a microcentrifuge to pellet the cells. The supernatant was decanted and the cells were re-suspended in 1 mL of reagent for isolation of total RNA (TRI Reagent Solution; Life Technologies). The tubes were stored at 4°C until RNA extraction. This experiment was stopped after the 4 hour cell

viability check because more than 50% of the cells were not viable. The method was modified and is described below.

2.5 Heat Course Trials for RNA Extraction – Version 2

DFTD cells (C5065 cell line) that had been growing in culture were collected, centrifuged at 300 x g for 10 minutes at room temperature, and re-suspended in 6.5 mL of complete media [RPMI medium (RPMI 1640, USA) + 10% fetal calf serum + 5% GlutaMAX + 5% Antibiotic-antimycotic (Anti-Anti); Gibco, USA]. Six wells in a cell culture cluster plate were filled with 800 µL of this solution each. An additional 1 mL of complete culture media was added to each of the wells. These were incubated overnight in an incubator set to 35°C. Each of the wells of cells was designated to be heated for a certain number of hours: one for zero (0) hours, one for two (2) hours, one for four (4) hours, one for eight (8) hours, one for eighteen (18) hours, and one for twenty-four (24) hours. All of the wells except the 0 hour one were placed in an incubator set to 40°C. The wells were individually taken out of the incubator after they incubated for their designated amount of time, staggering the rest of the procedure; for the 0 hour well, the following steps were started immediately after removal from the 35°C incubator. The cell viability for each well was checked (all were >95% viable). The cells were then washed from the bottom of the wells with 2 mL of new complete media and transferred to new 2 mL tubes (Eppendorf Safe-Lock Microcentrifuge Tubes). The tubes were then centrifuged at 14,000 rpm in a microcentrifuge to pellet the cells. The supernatant was decanted and transferred into individual tubes, and the cells were re-suspended in 1 mL of reagent for isolation of total RNA (TRI Reagent Solution; Life Technologies). The tubes were stored at 4°C until RNA extraction.

2.6 RNA Extraction

A total of 0.2 mL of chloroform was added to the tubes with samples obtained in the heat course trial, and the tube was shaken vigorously to combine. The samples were incubated for 10 minutes at room temperature to allow the phases to settle, and they were centrifuged at 4°C at 12,000 x g for 15 minutes. The aqueous phases of the samples were removed and placed into new individual tubes (Eppendorf Safe-Lock Microcentrifuge Tubes). To isolate the RNA, 0.5 mL of 100% isopropanol was added to each tube and the tubes were inverted to mix. The tubes were incubated at room temperature for one hour and were centrifuged at 12,000 x g for 20 minutes at 4°C to pellet the RNA. To wash the RNA, the supernatant was removed from the tubes, leaving only the RNA pellets. The pellets were washed with 1 mL of 75% ethanol. The samples were then vortexed briefly and centrifuged at 7,500 x g for 5 minutes at 4°C. The supernatants were discarded, and the wash procedure was repeated. The ethanol was then pipetted off and the pellets were allowed to dry for 10 minutes. The RNA pellets were then re-suspended in 50 µL of TE buffer (Tris-EDTA Buffer; Life Technologies) by passing the solution up and down several times through a pipette tip. The tubes were incubated in a heating block at 60°C for 15 minutes to completely dissolve the RNA. Next, 5 µL of a DNase buffer (10X TURBO DNase Buffer; Life Technologies) and 1 µL of a DNase (TURBO DNase; Life Technologies) were added to 50 µL of the RNA and mixed gently. The tubes were incubated at 37°C for 30 minutes. A total of 5 µL of re-suspended DNase inactivation reagent (DNase Inactivation Reagent; Life Technologies) was added and the solutions were mixed well. The tubes were then incubated at room temperature for 5 minutes, being flicked every 2 minutes. The tubes were then centrifuged at 10,000 x g for 1.5 minutes to pellet the reagent, and the RNA suspensions were transferred to new tubes. The amount and purity of the RNA was quantified

using a Nanodrop; the machine was initialized with 2 μL of water and was blanked with 2 μL of TE buffer. Then, 2 μL of each RNA sample were placed on the Nanodrop individually for quantification. The RNA samples were then diluted to a concentration of 100 ng/ μL . Next, in 200 μL tubes (0.2 mL Individual Thin-Walled PCR Tubes; Bio-Rad) on ice, a template-primer mix was prepared for each reaction by adding the following components in the order listed: 5 μg total RNA in a 9 μL volume of TE buffer and 1 μL Oligo(dT)₁₅ primer [Oligo(dT)₁₂₋₁₈ Primer; Life Technologies]. An additional reaction was included as a “no reverse transcriptase control” for identification of any contaminating DNA. The template-primer mixtures were denatured in a thermal cycler with a heated lid by heating the samples for 5 minutes at 70°C and then cooling for 5 minutes at 4°C. The tubes were centrifuged and placed on ice. A reaction master mix was then prepared in an Eppendorf tube for the samples by adding the following components in the order listed: 25.5 μL of nuclease-free water, 68 μL of reaction buffer (GoScript 5X Reaction Buffer; Promega), 34 μL of MgCl₂, 17 μL of PCR nucleotide mix (PCR Nucleotide Mix 10 mM; Promega), 8.5 μL of ribonuclease inhibitor (Recombinant RNasin Ribonuclease Inhibitor; Promega), and 1 μL of reverse transcriptase (GoScript Reverse Transcriptase; Promega) to all samples except the control, where water was added instead. The internal control used was 18S rRNA because it shows low variance in expression. Then, 10 μL of the master mix was pipetted into the appropriate template-primer mixes for a final reaction volume of 20 μL . This was mixed gently and centrifuged briefly. The samples were then placed in a thermal block cycler with a heated lid at 25°C for 5 minutes, 42°C for one hour, 72°C for 15 minutes, and 4°C hold. The reaction was stopped by placing the tubes on ice.

2.7 Quantitative Reverse Transcriptase PCR of RNA Samples

A quantitative PCR was performed on the RNA extracted from the heat shocked DFTD cells with specific primers for HSP 27, HSP 60, HSP 70, and HSP 90 (Appendix 1, Table A2). The PCR primers used were diluted to 5 μ M with nuclease-free water. A PCR master mix was prepared for 21 reactions with 10 μ L each total. The following were added together in order: 105 μ L of hot start reaction mix (FastStart DNA Master SYBR Green; LightCycler), 12.6 μ L of forward primer (5 μ M), 12.6 μ L of reverse primer (5 μ M), and 37.8 μ L of PCR-grade water. The master mix was mixed carefully and was briefly centrifuged. Then, 8 μ L of the master mix were pipetted into each well of a 96-well PCR plate. Next, 2 μ L of the appropriate cDNA samples were added to the appropriate wells. The plate was covered with self-adhesive foil and briefly centrifuged to pool the samples at the bottom of the wells. The program LightCycler 480 SW 1.5 was used for the analysis with the following detection format settings: SYBR Green 1/HRM Dye/Block Size 96/Reaction Volume 10. The experiment was started with pre-set settings. Cycle threshold values were recorded and melt curves were obtained.

2.8 Western Blot on Supernatant Samples

Western blots were performed on the supernatant samples from the heat course trial to investigate whether heat shock proteins 27, 70, and 90 were released into the supernatant during heat shocking. Protein from the supernatant samples was prepared with LDS buffer, a reducing agent, and deionized water. A total of 10 μ L of this solution was loaded into each well of three gels (4%-12% Bis-Tris Protein Gel; Life Technologies); one for HSP 27, one for HSP 70, and one for HSP 90. A pre-stained protein ladder was also run in a lane on each of these gels. The gels were allowed to run with MES buffer for 35 minutes at a constant voltage of 165 V. The gels were then dry-transferred onto nitrocellulose membranes for 7.5 minutes (iBlot Dry Blotting

System; Life Technologies). The membranes were blocked for one hour at 4°C in blocking buffer (Animal-Free Blocker; Vector Laboratories) and were rinsed once with TBS-Tween. The membranes were then incubated in 50 mL tubes (50 mL Centrifuge Tubes; Corning) at room temperature with the appropriate primary antibodies diluted in blocking buffer. The primary antibodies used were as follows: HSP 27: mouse anti-human HSP 27 (1/1,000), HSP 70: mouse anti-human HSP 70 (1/1,000), HSP 90: rabbit (serum) anti-human HSP 90 (1/20,000). After this incubation, the membranes were washed a total of four times in TBS-Tween: once for ten minutes and three times for five minutes each. The membranes were then incubated at room temperature with the appropriate secondary antibodies diluted in blocking buffer. The secondary antibodies used were as follows: HSP 27: goat anti-mouse HRP (1/1,000), HSP 70: goat anti-mouse HRP (1/4,000), HSP 90: goat anti-rabbit HRP (1/4,000). The membranes were washed four times as described before. The membranes were then stained with a chemiluminescent substrate solution (Immobilon Western Chemiluminescent HRP Substrate; Merck Millipore) and incubated for 5 minutes. Finally, the membranes were imaged for 2 minutes each using Carestream Molecular Imaging with the setting Chemiblot: standard signal.

2.9 Heat Course Trials for Protein Extraction

The DFTD cells (C5065 cell line) that were used for this experiment were grown in culture, injected into a mouse to grow as a tumour, and then harvested and grown in cell culture flasks. For this heat course trial, eight different lengths of incubation time were used. The DFTD cells were collected from the flask in which they were growing and were split into eight small flasks (CellBIND® Surface Cell Culture Flasks; Corning) with 5 mL of complete media [RPMI medium (RPMI 1640, USA) + 10% fetal calf serum + 5% GlutaMAX + 5% Antibiotic-antimycotic (Anti-Anti); Gibco]. Each of the flasks of cells was designated to be heated for a

certain number of hours: one for zero (0) hours, one for two (2) hours, one for four (4) hours, one for six (6) hours, one for eight (8) hours, one for nine (9) hours, one for eighteen (18) hours, and one for twenty-four (24) hours. All of the flasks except the 0 hour one were placed in an incubator set to 40°C. The flasks were individually taken out of the incubator after they incubated for their designated amount of time, staggering the rest of the procedure; for the 0 hour flask, the following steps were started immediately after harvesting the cells. The cell viability for each flask was checked (all were >90% viable). The cells were then washed from the bottom of the flasks with the existing media and were transferred to new 10 mL tubes (10 mL Graduated Centrifuge Tube; Techno Plas). The tubes were then centrifuged at 300 x g for 5 minutes, and the supernatant was discarded. The cells were washed twice in cold PBS and then were pelleted by centrifuging again at 300 x g for 5 minutes. The pellet was re-suspended in 1 mL cold PBS solution, and the cell suspension was transferred to a pre-weighted 1.5 mL tubes (Eppendorf Safe-Lock Microcentrifuge Tubes). The cell viability was then checked. The cells were pelleted for 60 seconds in a microcentrifuge at 14,500 rpm, and the supernatant was discarded. The weights of the wet cell pellets were recorded. The tubes were then submerged in liquid nitrogen and transferred to a -80°C freezer.

2.10 Protein Extraction

To extract the protein from the cell pellets, 280 µL of RIPA buffer (1X RIPA Lysis and Extraction Buffer; Thermo Scientific) were added to each cell pellet. The mixture was pipetted up and down to suspend the pellet. The pellets were then sonicated for 10 seconds with 50% pulse. The mixture was shaken gently for 15 minutes at 4°C and then centrifuged at 14,000 x g for 15 minutes at 4°C to pellet cell debris. The supernatants were then transferred to new clean tubes and the amount of protein in each sample was quantified.

2.11 Western Blot on Extracted Protein Samples

Western blots were performed on the protein samples from the heat course trial. Protein from the samples was prepared with LDS buffer, a reducing agent, and deionized water. A total of 10 μ L of this solution was loaded into each well of five separate gels (4%-12% Bis-Tris Protein Gel; Life Technologies); one for HSP 27, one for HSP 70, one for HSP 90, and two negative control gels: one for mouse IgG and one for rabbit IgG. A pre-stained protein ladder was also run in a lane on each of these gels. The gels were allowed to run for 35 minutes with MES buffer at a constant voltage of 165 V. The gels were then dry-transferred onto nitrocellulose membranes for 7.5 minutes (iBlot Dry Blotting System; Life Technologies). The membranes were blocked for one hour at 4°C in blocking buffer (Animal-Free Blocker; Vector Laboratories) and were rinsed once with TBS-Tween. The membranes were then incubated in 50 mL tubes (50 mL Centrifuge Tubes; Corning) at room temperature with the appropriate primary antibodies and the controls diluted in blocking buffer. The primary antibodies used were as follows: HSP 27: mouse anti-human HSP 27 (1/1,000), HSP 70: mouse anti-human HSP 70 (1/1,000), HSP 90: rabbit (serum) anti-human HSP 90 (1/20,000), Mouse IgG Control: mouse IgG (1/200), Rabbit IgG control: rabbit IgG (1/20,000). In addition, as controls, 2 μ L of alpha-tubulin mouse (1/5,000) were added to the tubes with the HSP 27 and HSP 70 membranes and 2 μ L of actin rabbit (1/5,000) were added to the tube with the HSP 90 membrane. After this incubation, the membranes were washed a total of four times in TBS-Tween: once for ten minutes and three times for five minutes each. The membranes were then incubated at room temperature with the appropriate secondary antibodies diluted in blocking buffer. The secondary antibodies used were as follows: HSP 27: goat anti-mouse HRP (1/5,000), HSP 70: goat anti-mouse HRP (1/5,000), HSP 90: goat anti-rabbit HRP (1/5,000), Mouse IgG Control: goat anti-

mouse HRP (1/5,000), Rabbit IgG Control: goat anti-rabbit HRP (1/5,000). The membranes were washed four times as described before. The membranes were then stained with a chemiluminescent substrate solution (Immobilon Western Chemiluminescent HRP Substrate; Merck Millipore) and incubated for 5 minutes. Finally, the membranes were imaged for two minutes each using Carestream Molecular Imaging with the setting Chemiblot: standard signal. The blots were then quantified by measuring the band intensities of HSP 70, HSP 90, actin, and tubulin using the ImageJ program.

2.12 Data Analyses

The relative expression of HSPs 27, 60, 70, and 90 according to the Quantitative PCR was assessed using a two-way analysis of variance (ANOVA) with incubation time and heat shock protein as the independent variables (Appendix 1, Table A1). $P < 0.05$ was considered statistically significant.

3.0 Results

3.1 Western Blot to Determine Presence of HSP 27 and HSP 70

The image taken of the Western blot for HSP 27 revealed no bands. To eliminate the possibility that this was due to human error such as faulty protein transfer onto the membrane, the Western blot only was repeated using the same membrane with antibodies specific to HSP 70. That image revealed eight bands equal in size across the gel at 70 kDa.

3.2 Quantitative Reverse Transcriptase PCR of RNA Samples

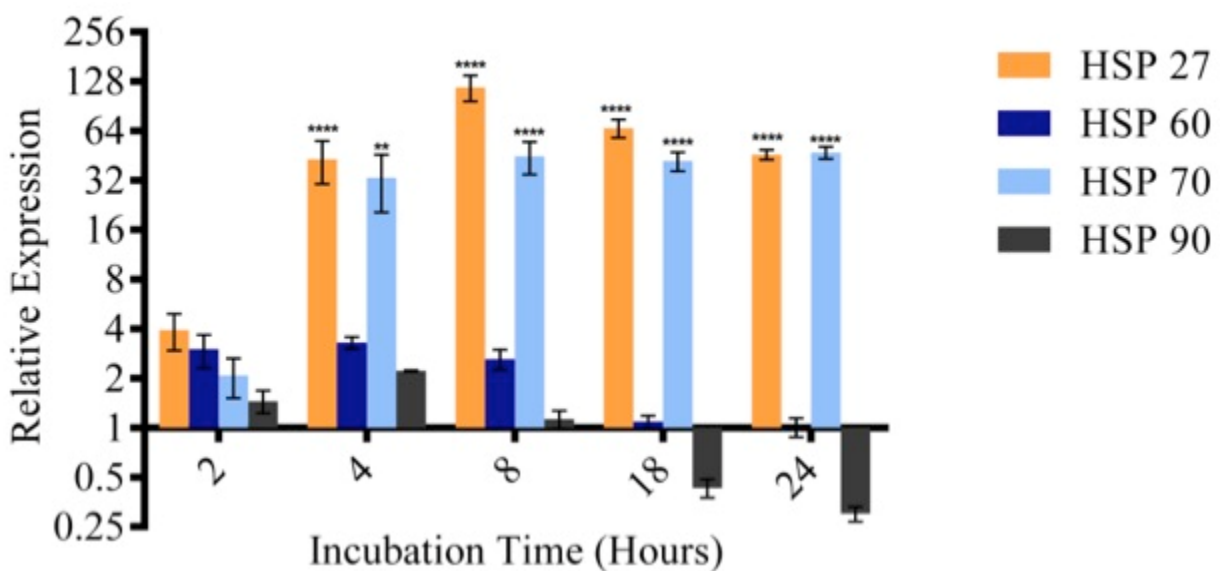


Figure 1 Relative expression of heat shock proteins 27, 60, 70, and 90 in DFTD cells represented as a fold-change when compared with untreated cells. All cells were incubated in an incubator at 40°C for varying lengths of time (0, 2, 4, 8, 18, or 24 hours). Bars represent \pm s.e. ****= $p \leq 0.0001$, **= $p \leq 0.01$, unlabeled=not significant.

Figure 1 shows the relative expression of RNA for specific HSPs from heat shocked DFTD cells as a fold-change when compared to untreated cells. Overall, RNA for HSP 27 had the highest relative expression of all of the heat shock proteins tested. Its relative expression was the highest at 8 hours of incubation, nearly 128-fold higher than that of the untreated cells. All of the incubation times tested on RNA for HSP 27, with the exception of 2 hours ($p > 0.05$), showed

significantly higher relative expression when compared with the untreated cells ($p \leq 0.0001$). RNA for HSP 60 saw minimal change in its relative expression throughout the entire heat course. Although its relative expression was highest at 4 hours, being nearly 4-fold higher than that of untreated cells, none of the incubation times showed a significant relative expression when compared with the untreated cells ($p > 0.05$). RNA for HSP 70 had the second highest relative expression overall. Its relative expression was the highest at 24 hours of incubation, nearly 64-fold higher than that of the untreated cells. The most significant fold-changes occurred at the 8, 18, and 24 hour incubation times ($p \leq 0.0001$), but a significantly higher relative expression was also seen after 4 hours ($p \leq 0.01$). Only the 2 hour incubation time did not show a significant fold-change when compared with the untreated cells ($p > 0.05$). Finally, RNA for HSP 90 seemed to show the least change in relative expression. None of the incubation times tested showed a significant relative expression when compared with the untreated cells ($p > 0.05$). After 18 and 24 hours, HSP 90 even had lower relative expression than did the untreated cells.

3.3 Western Blot on Supernatant Samples

The images taken of the Western blot on the supernatant samples with HSP 70 and HSP 90 both showed a row of very intense bands around 55 kDa, while the image of HSP 27 showed faint bands in the same area. No other bands were detected in any of the images.

3.4 Western Blot on Extracted Protein Samples

The image taken of the Western blot with HSP 27 showed one row of bands at around 55 kDa that were all even in intensity. The image taken of the Western blot with HSP 70 also showed one row of bands at around 55 kDa, but another fainter row of bands appeared in this image at around 70 kDa. Both rows of bands seemed to be decreasing in intensity across the length of the membrane. The image taken of the Western blot with HSP 90 showed one row of

bands around 45 kDa that decreased slightly in intensity across the membrane as well as a fainter row of bands around 90 kDa that also seemed to decrease slightly in intensity across the membrane. The image of the Mouse IgG control Western blot showed no bands, indicating that there was no non-specific binding of the antibodies. The image of the Rabbit IgG control Western blot showed some bands across the membrane, indicating the presence of some non-specific binding of the antibodies.

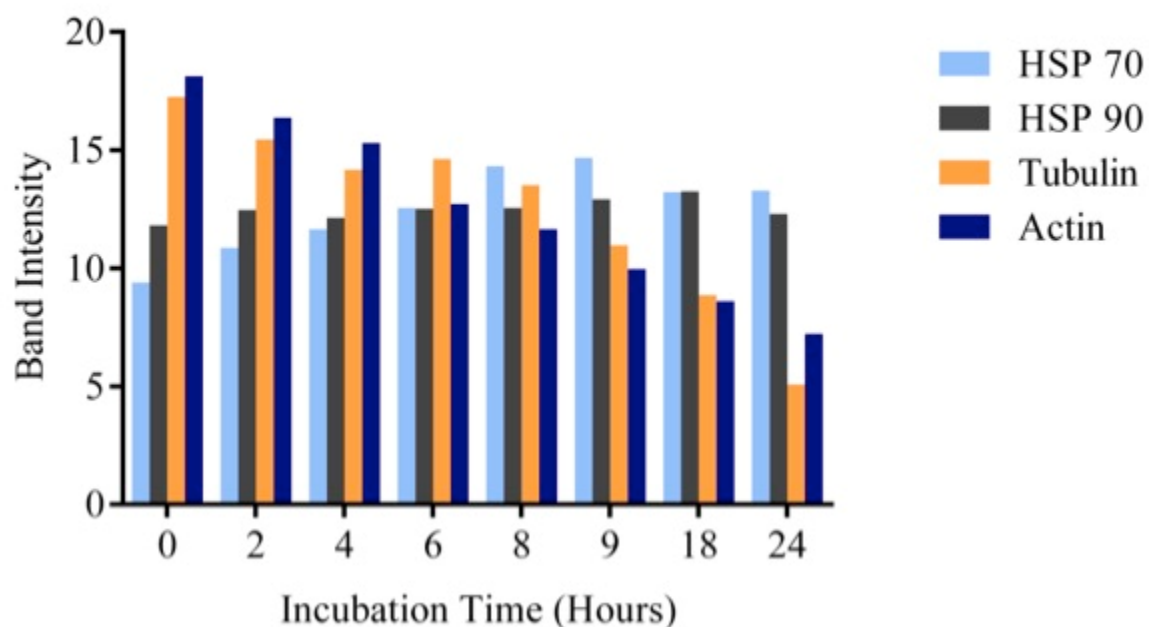


Figure 2 Band intensity revealing expression of heat shock proteins 70 and 90 from DFTD cells as well as tubulin and actin controls; represented as percentages of the total intensity of the bands in a particular membrane. All cells were incubated in an incubator at 40°C for varying lengths of time (0, 2, 4, 6, 8, 9, 18, or 24 hours).

The ImageJ band intensity quantification revealed the intensity of each of the bands in the HSP 70 and HSP 90 Western blot images in the form of percentages of total intensity of the bands in a particular membrane (Figure 2). For HSP 70, the general trend was a steady increase in band intensity followed by a slight decrease after 9 hours. The most intense band occurred at 9 hours, where it made up 14.3% of the total band intensity. The least intense band occurred at 0

hours, where it made up only 9.4% of the total band intensity. For HSP 90, there was very little variation in band intensity across the incubation times tested. The most intense band occurred at 18 hours with 13.3% of the total band intensity, but the least intense band was hardly smaller: 11.3% of the total band intensity, occurring at 0 hours. Tubulin showed a steady decrease in band intensity, with the highest intensity occurring at 0 hours (17.3% of the total band intensity) and the lowest intensity occurring at 24 hours (5.1% of the total band intensity). Actin followed a similar general trend in decreasing band intensity; the highest band intensity was at 0 hours (18.1% of the total band intensity) and the lowest band intensity was at 24 hours (7.2% of the total band intensity).

4.0 Discussion

4.1 Summary of Results

This study revealed limited information about the expression of HSP 27 in DFTD cells because it did not appear on any Western blot performed; it was, however, shown to have significantly increased relative expression of RNA after being heat shocked for several hours. RNA for HSP 60 was not found to be significantly expressed even after being heat shocked, and it was not possible to test the actual protein expression. HSP 70 expression increased significantly after being heat shocked for several hours. Accordingly, the relative expression of RNA for HSP 70 was found to be increased as heat shocking time increased. Finally, HSP 90 showed little variation in protein expression even after heat shocking, and the same was found to be true of relative expression of RNA for HSP 90.

4.2 Western Blot to Determine Presence of HSP 27 and HSP 70

The absence of bands in the Western blot for HSP 27 is unexpected because HSP 27 is known to be present in *S. harrisii* (Ensembl, 2014). This could be due to several factors; one possibility is that either the primary or the secondary antibodies were not concentrated enough and that they were not binding effectively to HSP 27. The more likely explanation, however, is that the primary antibody used did not recognize HSP 27 in devils, meaning that it simply does not react with the target protein as it would in other species. The proper appearance of HSP 70 bands on the same membrane seemed to eliminate human error, such as poor transfer to the membrane or excessive washing of the membrane, as a reason for the lack of a signal from HSP 27. It would be valuable to repeat this experiment with a different antibody that would perhaps better react with HSP 27 from DFTD cells.

4.3 Quantitative Reverse Transcriptase PCR of RNA Samples

The quantitative reverse transcriptase PCR of the RNA extracted from the heat shocked cells showed that overall relative RNA expression was the highest in HSP 27 followed closely by HSP 70. Because HSPs 27 and 70 both function as chaperone proteins that rescue proteins from denaturing due to stressful environmental conditions (Lamb et al., 1989), it is logical that their RNA expression increased with change in an environmental stressor: increasing temperature. HSPs 60 and 90 showed no significant fold-increase in relative RNA expression when compared with untreated cells; it is interesting that the cells were not dying despite the fact that certain heat shock proteins were not being upregulated to help protect other proteins in the cells that were denaturing from the heat. It is possible that higher heat or different incubation times would result in higher expression of RNA; a study conducted by Lang et al. (2000) used slightly different methods to heat shock cells than did this study. Cells were incubated at 47°C in a water bath for

just 20 minutes. The researchers found that heat shock significantly increased the relative RNA expression of HSP 70. This is consistent with the results of this study, but it provides valuable information on alternate methods of heat shocking that could enhance the relative expression of RNA for heat shock proteins that were not significantly upregulated in this study, including HSPs 60 and 90.

4.4 Western Blot on Supernatant Samples

The Western blot performed on the supernatant samples from the heat course trial did not provide much valuable information for this study. The bands seen on all three membranes at around 55 kDa are mostly likely just signals from fetal calf serum that was present in the cell culture media. Fetal calf serum is known to have a molecular weight of ~51 kDa (Invitrogen, n.d.), which validates this claim. There are several possible explanations for the absence of other bands on all of the membranes; one is that the signal for fetal calf serum was so strong during imaging that the signals for the HSPs of interest were overshadowed and effectively eliminated from the image. Another possibility is that the heat shock proteins simply were not released in large concentrations into the supernatant during heat shocking. According to research conducted by Basu et al. (2000), it is possible that the heat shock proteins were just not released into the supernatant. Their study showed that necrotic cell death, but not apoptotic cell death, results in the release of HSP 70 and HSP 90 into the supernatants. Because the cell viability of all of the samples after heat shocking was >95%, it is possible that there was a low level of cell death in culture and thus a low level of HSP release. Further, Lang et al. (2000) found that exposing cells to heat shock resulted in a rapid and significant upregulation of HSP 70 with no injury to the cell and without apoptosis occurring.

4.5 Western Blot on Extracted Protein Samples

The general trends for HSP 70 and HSP 90 observed in the graph of protein band intensities (Figure 6) agreed with the general trends seen for the same proteins in the graph of relative RNA expression (Figure 1). In Figure 1, relative RNA expression for HSP 70 was seen to increase significantly after the 2 hour incubation time point. Similarly, protein expression of HSP 70 seen in Figure 6 increased steadily to a maximum at 9 hours and then dropped off only slightly. This shows that incubation at 40°C for increasing lengths of time did have an upregulatory effect on HSP 70. This result is perfectly consistent with the results from a study conducted by Dokladny et al. (2006); the researchers found that HSP 70 was increasingly expressed as heat shock incubation time increased. According to this study, HSP 70 is most upregulated after 9 hours of incubation at 40°C.

In general, HSP 90 showed very little change in protein expression throughout the heat course (Figure 6). This was also seen in the graph of relative RNA expression, where none of the time periods showed a significant fold-change in relative RNA expression. These results differ from results obtained by Dokladny et al. (2006) in a similar study on intestinal epithelial cells, which found that increasing time periods of heat shocking was linked with a significant increase in the expression of HSP 90. Perhaps this difference can be attributed to DFTD cells having a lower initial concentration of HSP 90 than do intestinal epithelial cells. Additionally, Dokladny et al. (2006) heated the cells at 41°C as opposed to the 40°C used in this study; it is possible that this accelerated the upregulation of HSP 90. HSP 90 was most upregulated at 18 hours, though only marginally.

It is important to note the obvious trend of decreasing protein expression with time in both the tubulin and the actin protein samples. The controls should have even band intensities

across the entire membrane, but this was not the case in this experiment. Although this could cause uncertainty about the credibility of the results, it should instead do the opposite. It would be expected that if the control bands were decreasing in intensity across the membrane because of some transfer error or other mistake, the sample bands would be decreasing in intensity as well. Instead, the sample bands for HSPs 27 and 70 increased steadily, and the band intensity for HSP 90 did not change significantly. The decrease in protein expression in the controls could thus be visually lessening the extent to which HSPs 27, 70, and 90 were upregulated. One explanation for the unusual decrease in expression of both tubulin and actin is that they began to be degraded over time while being heated in the heat course trial. A study conducted by Kramer et al. (2008) showed that the transcription of tubulin mRNAs is decreased by as much as 50% after heat shock, while the maturation of HSP 70 mRNA continues even more efficiently than before. This is consistent with what was found in this study, that tubulin RNA expression decreased over time with heat shocking and HSP 70 RNA expression increased with additional heat shocking. Another study that supports this theory was conducted by Prasad et al. (1998) and revealed that tubulin undergoes time-dependent decay, or a loss of its functional properties over time. This effect is especially amplified with heating, as was shown in this study.

4.6 Other Considerations

The vast majority of previous studies investigating the role of heat shock proteins in the immune system have been conducted *in vitro*, and the possibility of using them as adjuvants to a vaccine is still theoretical (Tobian et al., 2004). Heat shock protein behavior in laboratory settings may deviate from behavior when implanted *in vivo*.

5.0 Conclusion and Future Directions

This study aimed to show how heat shocking devil facial tumour disease cells affects the expression of heat shock proteins 27, 60, 70, and 90. HSPs 27 and 70 had significantly increased relative RNA expression after heat shocking for increasing lengths of time. As could be expected, the protein expression for HSP 70 also increased with length of incubation time. HSP 60 showed no significant relative expression of RNA even after heat shocking. Finally, HSP 90 showed insignificant variation in both protein expression and relative RNA expression, even after heat shocking.

Time was a limiting factor in this study. Further research is required to continue investigating the possibility of using heat shock proteins as adjuvants to an anti-DFTD vaccine. An additional heat course study will be conducted in the near future using a different antibody against HSP 27 to determine protein expression of HSP 27. The results should further support the findings in this study, that HSP 27 is effectively upregulated with heat shocking. Conducting this experiment is important, because the results of the present study showed HSP 27 as the most promising candidate to act as an adjuvant in a vaccine due to its significant increase in expression after heat shocking.

In addition, it would be valuable to test other methods for heat shocking, such as water baths for incubation, higher incubation temperatures, and even more variable lengths of time as did Lang et al. (2000). Testing a wider range of heat shock proteins would be valuable in finding the most potent inducer of immune function. There is further research currently being done at Menzies Institute for Medical Research studying toll-like receptor agonists and various anti-cancer drugs, all for the development of a functioning vaccine against DFTD.

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Appendix 1

Table A1 Summary of two-way ANOVA Dunnett's Multiple Comparisons Test comparing the relative expression of proteins across incubation time (T=0-24 hours) and gene (HSPs 27, 60, 70, 90). SS=sum of squares, DF=degrees of freedom, MS=mean square, F (DFn, DFd)=F distribution, P<0.05 considered significant.

Source of Variation	SS	DF	MS	F (DFn, DFd)	P value
Incubation Time	14418	5	2884	F (5, 10) = 26.28	P < 0.0001
Gene	26100	3	8700	F (3, 6) = 37.65	P = 0.0003
Incubation Time x Gene	20677	15	1378	F (15, 30) = 15.72	P < 0.0001

Table A2 Forward and reverse primers used for the quantitative reverse transcriptase PCR of RNA samples extracted from DFTD cells after heat shocking at 40°C for varying lengths of time (0-24 hours).

Gene	Forward Primer	Reverse Primer
18S rRNA	5' TCGGGACTGCTCTACAAACG 3'	5' ACCCTTGATGGCTGTGATGG 3'
HSP70 A2	5' GTATTGAAACCGCAGGGGGA 3'	5' AGACACTGCTCTGGTTGTCTG 3'
HSP60 D1	5' AGGCAAGGGTGAAAAATCCCA 3'	5' AAGCAATGCACAACCACCAC 3'
HSP90b (gp96)	5' TTGTTCCCACTTCTGCTCCC 3'	5' AGCCGAGTACGGTTGGAATG 3'
HSP27 (B1)	5' ACATTTGCTCGGTCCTCCC 3'	5' AAGCCGTGCTCATCTTGTCT 3'



ISP Ethics Review

(Note: Each AD must complete, sign, and submit this form for every student's ISP.)

The ISP paper by Monika Payerhin (student) does conform to the Human Subjects Review approval from the Local Review Board, the ethical standards of the local community, and the ethical and academic standards outlined in the SIT student and faculty handbooks.

Completed by: Tony Cummings

Academic Director: Tony Cummings

Signature:

A handwritten signature in black ink, appearing to read 'J. A. Cummings'.

Program: Australia: Rainforest, Reef, and Cultural Ecology

Date: 12/12/2014